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Seasonal and spatial dynamics of enteric viruses in wastewater and in riverine and estuarine receiving waters

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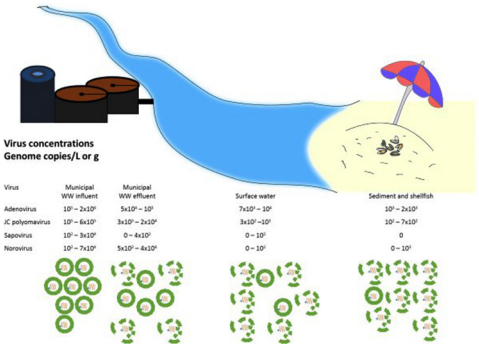
HIGHLIGHTS

- The TFUF concentration method is suitable for quantifying viruses in water samples.
- For the first time, sapovirus was found in UK waters.
- Enteric viruses were traceable from source to beaches and shellfish beds.
- Norovirus concentrations in the environment agreed with local outbreaks.
- PGM assay is useful to study norovirus degradation in the environment.

GRAPHICAL ABSTRACT



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ABSTRACT

Enteric viruses represent a global public health threat and are implicated in numerous foodborne and waterborne disease outbreaks. Nonetheless, relatively little is known of their fate and stability in the environment. In this study we used carefully validated methods to monitor enteric viruses, namely adenovirus (AdV), JC polyomavirus (JCV), noroviruses (NoVs), sapovirus (SaV) and hepatitis A and E viruses (HAV and HEV) from wastewater source to beaches and shellfish beds. Wastewater influent and effluent, surface water, sediment and shellfish samples were collected in the Conwy catchment (North Wales, UK) once a month for one year. High concentrations of AdV and JCV were found in the majority of samples, and no seasonal patterns were observed. No HAV and HEV were detected and no related illnesses were reported in the area during the period of sampling. Noroviruses and SaV were also detected at high concentrations in wastewater and surface water, and their presence correlated with local gastroenteritis outbreaks during the spring and autumn seasons. Noroviruses were also found in estuarine sediment and in shellfish harvested for human consumption. As PCR-based methods were used for quantification, viral infectivity and degradation was estimated using a NoV capsid integrity assay. The assay revealed low-levels of viral decay in wastewater effluent compared to influent, and more significant decay in

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environmental waters and sediment. Results suggest that AdV and JCV may be suitable markers for the assessment of the spatial distribution of wastewater contamination in the environment; and pathogenic viruses can be directly monitored during and after reported outbreaks to prevent further environment-derived illnesses.

1. Introduction

Enteric viruses are frequently associated with water- and foodborne disease outbreaks. Infected individuals typically shed enteric viruses at very high concentrations and hence these viruses are frequently found in wastewater (Okoh et al., 2010). Most enteric viruses are highly resistant to UV and chemical disinfection, therefore the methods routinely used in wastewater treatment plants may not greatly reduce the number of infectious viruses in the effluent (Sano et al., 2016) and these may enter the environment. Enteric viruses persist in the environment and often have very low infectious doses, leading to waterborne illness with outbreaks potentially occurring far from the source of contamination. Furthermore, viruses can accumulate in sediment and can be ingested by shellfish destined for human consumption, resulting in foodborne illnesses (Landry et al., 1983; Oliveira et al., 2011).

The viruses most commonly associated with waterborne diseases are adenovirus (AdV) group F, enterovirus A-D, hepatitis A and E viruses (HAV and HEV), norovirus genotype I and II (NoVGI and NoVGII), sapovirus genotype I (SaVGI), and rotavirus A (Ashbolt, 2015). In developed countries, the majority of water- and foodborne viral outbreaks, including those related to shellfish, are associated with noroviruses (NoVs) (Bosch et al., 2016; Rodríguez-Lázaro et al., 2012). Recently, other caliciviruses, the sapoviruses (SaVs), which have very similar structure to NoVs have also been shown to cause similar symptoms, mostly in young children. These have also been frequently detected in water and shellfish (Yates, 2014). In less economically developed countries, HAV and HEV are prevalent and, due to frequent global movement of people, these viruses are responsible for sporadic outbreaks worldwide (Dalton et al., 2013; Rodríguez-Lázaro et al., 2012). Due to the rapidly increasing number of cases associated with SaV and HEV worldwide, these viruses are considered emerging viruses (Sayed et al., 2015; Yates, 2014). While members of the caliciviruses, and HAV and HEV cause acute gastroenteritis, there are some enteric viruses, such as adenoviruses (AdVs) that are usually asymptomatic or result in mild illness. Furthermore, some commonly asymptomatic respiratory viruses e.g. polyomaviruses (PyV) are excreted in urine, and the possibility of gastroenteric transmission has been suggested (Bofill-Mas et al., 2001). These viruses circulate in the population and are frequently found in wastewater in high concentrations (Tran et al., 2015).

To evaluate the risk of faecal contamination in the environment, indicators, such as coliform bacteria and bacteriophages, are commonly used. However, their presence may not reflect the distribution of enteric viruses (Lin and Ganesh, 2013). Recently, several studies have directly monitored enteric viruses in the environment, mainly focusing on extreme weather events (Hata et al., 2014; Vieira et al., 2017) and on drinking water sources (Grøndahl-Rosado et al., 2014; Iaconelli et al., 2017).

The limited number of published spatiotemporal viral monitoring studies is partly due to the lack of reliable, standard methods for the detection of viruses in complex matrices, such as wastewater, river and lake water. In addition, evaluations of public health risk related to viral pollution in water require large volumes of water to be analysed. Traditional filtration methods used for water concentration are not suitable for high volume and/or high turbidity samples due to membrane clogging (Cashdollar and Wymer, 2013). In tangential flow ultrafiltration (TFUF), the filter membrane is parallel to the flow stream, hence only a proportion of the feed stream passes through the membrane (permeate) with the remainder (retentate) recirculating. In many environmental studies, TFUF approaches are used as they are superior for the filtration of high turbidity lake and seawater samples compared to other filtration-based methods (Francy et al., 2013; Sun et al., 2014).

Due to their sensitivity, accuracy and flexibility, PCR-based methods are used for the quantification of viruses, however, these assays only detect a short segment of the genome and the results provide no measure of viral infectivity (Ronnqvist et al., 2014). Furthermore, PCR amplification may be inhibited by organic matter (e.g. humic substances) co-extracted with nucleic acids and hence may give false negative results (Meschke and Sobsey, 1998; Rock et al., 2010).

The aim of the current study was to assess the fate and spatial and temporal distribution of several enteric viruses (NoV, SaV, HAV, HEV, AdV and PyV) in wastewater and surface water, along with sediment and shellfish samples derived from areas affected by wastewater contamination. For water concentration, a carefully validated TFUF technique was used coupled with PCR-based assays for virus quantification. To overcome the limitations of PCR-based detection, NoV particle integrity in the environment was also addressed using a capsid integrity assay.

2. Materials and methods

2.1. Target viruses and spiking

In this study, common and emerging RNA viruses (NoVGI and NoVGII, SaVGI, HAV and HEV) along with potential enteric virus indicators (human AdVs and two common PyV strains: BK and JC) were tested. Samples were spiked with approx. 10^6 mengovirus (MgV) particles, which is commonly used as a molecular process control to validate nucleic acid extraction and q(RT)-PCR. For method validation, 10 L of surface water samples were spiked with approx. 10^6 selected enteric virus particles (NoVGII, SaVGI, HAV, HEV and AdV type40).

The AdV40 (PHE-0108056v) cultured in HEK293 was kindly provided by Lydia Drumwright (University of Cambridge). The MgV strain VMC0 and HAV strain pHM17543c were kindly provided by Dr. James Lowther (Centre for Environment Fisheries and Aquaculture Science; CEFAS, UK). Sapovirus GI.2 was obtained from clinical stool samples and genotyped by the National Reference Centre for Gastroenteritis Viruses, Dijon, France. The viral sample was generated by the preparation of a 10% solution, using phosphate-buffered saline (PBS; pH 7.4), which was subsequently filtered through a 0.2-μm filter. Norovirus sample (diluted and filtered faecal matter from a patient with confirmed NoV GII infection) was provided by Dr. Lydia Drumwright (University of Cambridge, UK).

2.2. Sampling sites and sample collection

For the validation of the water concentration method, treated and untreated wastewater, river, estuarine and seawater samples were collected in the Conwy River and estuary (North Wales, UK) as shown in Fig. 1. Grab samples were collected at the four major wastewater treatment plants (WWTPs). Llanrwst and Betws-y-Coed WWTPs discharge to the Conwy and Llugwy rivers, the Llugwy joining the Conwy between Betws-y-Coed and Llanrwst. Tal-y-Bont WWTW discharges to the Conwy estuary. The Betws-y-Coed and Tal-y-Bont WWTPs use activated sludge as secondary treatment and serve ca. 1200 and 1000 inhabitants, respectively. The Llanrwst WWTP uses filter beds for secondary treatment and serves ca. 4000 inhabitants. No disinfection is used prior to discharge at Betws-y-Coed, Llanrwst and Tal-y-Bont WWTPs. The Ganol WWTP, serving ca. 82,000 inhabitants, uses filter beds for

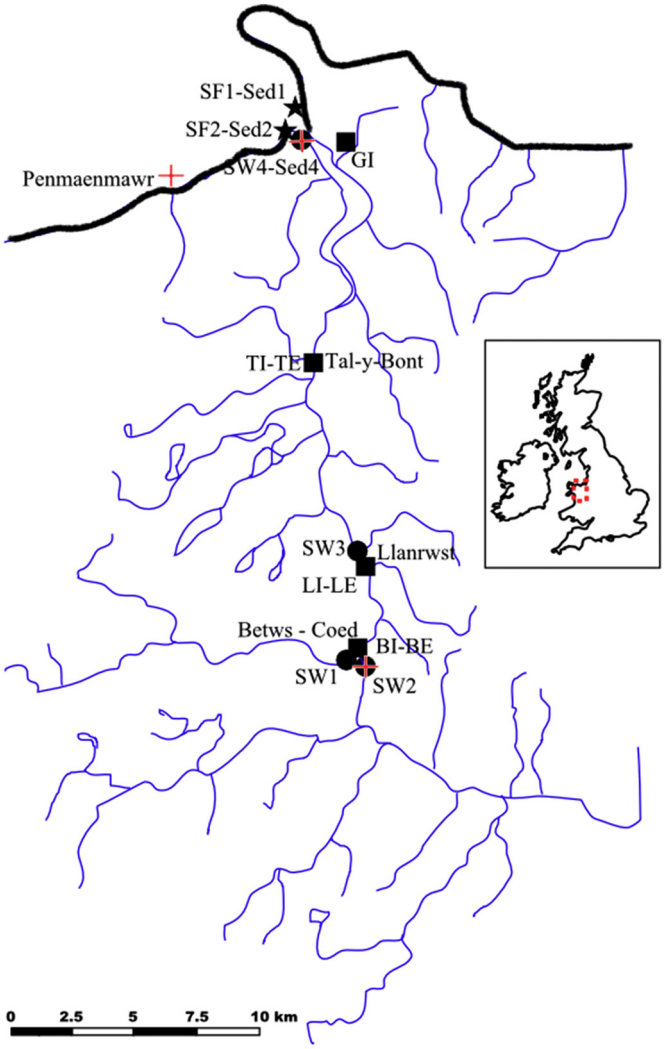


Fig. 1. Map representing the Conwy catchment and estuary, North Wales, with sampling sites for wastewater (square), surface water (circle) and sediment and shellfish (star). + indicates surface water sampling sites used for the spiking experiment.

secondary treatment followed by UV treatment. Although the treated wastewater from Ganol is discharged to the open sea, combined sewer outflows (CSOs) discharge directly to the Conwy estuary during heavy rainfall events affecting the water quality of the estuary. Mean flow rates are 614 m^3/day for Betws-y-Coed, 1142 m^3/day for Llanrwst, 763 m^3/day for Tal-y-Bont and 19,173 m^3/day for Ganol WWTP (data provided by Welsh Water, UK, 2016). By comparison, the median river flows at Betws-y-Coed and Llanrwst WWTWs are 1.15 and 4.81 m^3/s, giving indicative flow ratios river/WWTW of ~160 and ~360. Flow measurements for the four WWTPs do not include any release from CSOs as these are not gauged. The exact operation of the WWTPs under high flow conditions is not known with certainty, but each includes holding tanks to accommodate excess influent for later treatment, and CSOs. Under high flow conditions the ratio of river flow to total WWTW effluent is likely to be much greater than the average figures quoted above. At each WWTP, 1 L of influent and effluent samples were collected in duplicates in March 2016, during a local gastroenteritis outbreak. During the outbreak, two care homes with 36 individuals were affected (Public Health Wales, personal communication). However, the number of cases may have been much higher as most patients suffering from NoV do not visit a doctor. For method validation, 40 L of surface water was collected at Betws-y-Coed (river water; SW2), Conwy (estuarine water; SW4) and Penmaenmawr (seawater) at low tide (Fig. 1).

For the surveillance of enteric viruses, wastewater, surface water, sediment and shellfish samples were collected from August 2016 to August 2017. Rainfall was observed before/during the sampling of November 2016, January, February, July and August 2017. Wastewater samples, kindly provided by Welsh Water, UK, were collected at the WWTPs described above. At Betws-y-Coed, Llanrwst and Tal-y-Bont, 1 L influent and effluent samples were obtained (BI, BE; LI, LE; TI; TE), whereas at Ganol, 1 L influent samples (GI) were collected. Surface water grab samples of 10 L were collected from the Llugwy (SW1) and Conwy (SW2) rivers at Betws-y-Coed, upstream of the major wastewater treatment plants, at the tidal limit at Llanrwst (SW3) and in the estuary at Conwy (SW4) (Fig. 1). The SW2 samples were taken from a bridge whereas the other surface water samples were taken from the shore. Shellfish samples (SF1 and SF2), 20 blue mussels (*Mytilus edulis*) of various sizes, were hand-picked at the two commercial shellfish harvesting areas in the estuary. Together with the estuarine water sample and the shellfish samples, 10 g of the top 1 cm layer of sediment was also collected (Sed1, Sed2 and Sed4). All surface water, sediment and shellfish samples were collected at low tide. The pH and turbidity (T) of the wastewater and surface water samples were measured along with the conductivity (K) of the surface water samples.

2.3. Concentration of surface water and wastewater for virus detection

Prior to concentration, MgV was added to the samples as a whole process control. TFUF using 100 kDa mPES MiniKros® hollow fiber filter module with surface area of 790 cm^2 with the KrosFlo® Research IIi Tangential Flow Filtration System (Spectrumlabs, USA) was set up. Retentate was collected in a 250 mL reservoir. The system was washed with 2 L MilliQ water, and then a membrane integrity test was performed prior to each experiment according to the manufacturer's instructions. For blocking, the membrane was washed with 1 L of 0.01% sodium polyphosphate as described previously (Hill et al., 2005). After each run, the membrane was washed and disinfected by the circulation of Virkon® solution (Lanxess, Germany) for 5 min. Virkon® was washed out using MilliQ water. In order to reduce the costs, the membranes were reused. Prior to reuse, 100 mL of MilliQ water was circulated for 5 min and then collected. One membrane was dedicated to use for surface water samples and another membrane was strictly used for wastewater samples. Membranes were stored at 4 °C in 20% ethanol and reused for up to one year, if they passed the membrane integrity test.

During the experiments the inlet flow was set to 1000–1680 mL/min with 5 psi (0.3447 bar) pressure to achieve a permeate flow of 200–300 mL/min. Filtration was continued until approx. 5 mL sample remained in the reservoir. Then the flow was set to 680 mL/min with no pressure applied and the concentrate was circulated for 5 min with the permeate clamp closed. In order to increase recovery, the membrane was backwashed using approx. 20 mL 0.01% sodium polyphosphate solution added through the permeate pressure valve. The concentrate was collected from the system by introducing air through the retentate port. The final volume of the concentrate was approx. 50 mL.

The TFUF concentrates were further concentrated as described in Farkas et al. (2017a). Samples were mixed with beef extract and $NaNO_3$ to reach a final concentration of 3% and 2 M, respectively. The pH of the solution was adjusted to 5.5 and the sample was incubated for 30 min and then the solid matter was removed by centrifugation at 2,500 ×*g*, 10 min. The pH of the eluent was adjusted to 7.5 and incubated in 15% polyethylene glycol 6000 (PEG6000) and 2% NaCl overnight at 4 °C and centrifuged at 10,000 ×*g* for 30 min at 4 °C. The resulting pellet was resuspended in 2 mL phosphate-buffered saline (PBS; pH 7.4). The viral concentrates were stored at 4 °C for up to 24 h and then stored at −80 °C.

The solutions derived from the last washing step prior to the reuse of the membranes during the TFUF concentrations were concentrated by incubation in 15% PEG6000 and 2% NaCl overnight at 4 °C followed by centrifugation at 10,000 ×*g* for 30 min at 4 °C. The viral nucleic acids

were extracted directly from the pellet. All wash samples derived from the validation experiments were analysed, and two randomly selected samples were processed during each sampling event of the surveillance experiment.

2.4. Extraction of viral particles from sediment

Sediment samples were processed using beef extract elution (10 g sediment was mixed with 3× volume 3% beef extract in 2 M $NaNO_3$, pH 5.5) followed by PEG precipitation as described above. Further details on the validation of this method are described in Farkas et al. (2017a). MgV was added to the samples as a whole process control.

2.5. Extraction of viral particles from shellfish

Enteric viruses were extracted from the digestive tissue of the mussels following the standard method ISO/TS 15216-1:2013. In brief, 2 g aliquot of digestive tissue derived from 20 individuals was spiked with MgV as an extraction control and then incubated in proteinase K solution for 60 min at 37 °C followed by 15 min at 60 °C. The liquid phase was separated using centrifugation. The viral eluents were stored at 4 °C for up to 24 h and then stored at −80 °C.

2.6. Nucleic acid extraction

Viral nucleic acids were extracted from a 0.5 mL aliquot of the viral concentrates immediately after concentration using the NucliSENS® MiniMag® Nucleic Acid Purification System (bioMérieux SA, France). The final volume of the nucleic acid solution was 0.1 mL. When nucleic acids were extracted from the pellet the lysis buffer was added directly to the precipitate.

2.7. Quantification of viral nucleic acids

The RNA of NoVGI, NoVGII, HAV, HEV, SaVGI and MgV was quantified using two triplex TaqMan one-step qRT-PCR assays as described in Farkas et al. (2017b). Human AdV DNA was quantified using a SYBR Green qPCR assay. The reaction mix contained 1× QuantiFast SYBR Green PCR Mix with 10 pmol of the forward (CATGACTTTTGAGGTG GATC), 10 pmol of the reverse (CCGGCCGAGAAGGGTGTGCGCAGGTA) primers (van Maarseveen et al., 2010), and 1 μg bovine serum albumin (BSA). Amplification of the AdV target sequence was carried out using the following thermal cycling conditions: 95 °C for 5 min, then 40 cycles of 95 °C for 15 s, 55 °C for 1 min. Amplification was followed by one cycle of melting curve analysis at 95 °C for 15 s; annealing 60 °C for 1 min. Dissociation was carried out from 60 °C to 95 °C with a temperature ramp of 0.05 °C/s. Analysis indicated a melting peak (Tm) at 86.2 °C ± 0.3 °C. Amplification with different melting temperature was considered negative. The JC and BK polyomavirus (JCV and BKV) were quantified using TaqMan qPCR assays as described in Chehadeh and Nampoory (2013).

The q(RT)-PCR assays were carried out in a QuantStudio® Flex 6 Real-Time PCR System (Applied Biosystems, USA). For quantification, dilution series of a plasmid DNA carrying the target sequence were used. For all samples 2–8 μL of the original and a ten-times (vol:vol) diluted extract were tested. The viral concentrations were expressed as genome copies (gc)/L or gc/g.

2.8. Preparation of PGM-MGs for NoV capsid integrity

NoV capsid integrity was assayed using porcine gastric mucin-conjugated magnetic beads (PGM-MBs). This protein harbours the H1, A and Lewis b antigens, which are natural ligands for NoV particles (Cheetham et al., 2007; Tian et al., 2005). The mucin was covalently bonded to the beads using a two-step EDAC (1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride) - sulfo-NHS (N-hydroxysulfosuccinimide) method. A 0.5 mL aliquot of MagnaBind Carboxyl Derivatized Beads (Thermo Fisher Scientific, USA) were washed three times with PBS and incubated in 10 mg/mL EDAC and 10 mg/mL sulfo-NHS in 50 mM 2-(4-morpholino)-ethane sulfonic acid (MES), 0.9% NaCl (pH 4.7) conjugation buffer for 30 min at room temperature. The beads were then resuspended in conjugation buffer containing 5 mg/mL PGM (M1778, Sigma, USA) and gently agitated at room temperature for 14 h. The coated beads were then washed three times with PBS and resuspended in 0.5 mL PBS. PGM-MBs were stored at 4 °C and used within 4 months.

2.9. NoV capsid integrity assay for environmental concentrates

The concentrated surface water samples used in the spiking experiment (Section 2.1) were subjected to the PGM assay. A 0.1 mL aliquot of the samples was mixed with 370 μL PBS and 30 μL PGM-MGs for 30 min at room temperature. The PGM-MGs were separated using a magnetic bead attractor and then washed and resuspended in 0.5 mL PBS. The sample was then incubated at 96 °C for 5 min. The PGM-MGs were separated using a magnetic bead attractor and the viral RNA was extracted from the supernatant as described above. The final volume of the RNA solution was 50 μL. NoV RNA was quantified using qRT-PCR as described above.

Viral concentrates that were positive for NoV were five-times diluted in PBS prior to evaluating capsid integrity assay. A 0.1 mL aliquot of those samples was subjected to the PGM-MG assay as described above. Test volume was increased 5× for low concentration samples.

2.10. Data analysis

The limit of detection (LOD) of the processes were 25 gc/L in wastewater, 10 gc/L in surfaces water, 10 gc/g in sediment and 25 gc/g in shellfish. The limit of quantification (LOQ) of the processes was approx. 200 gc/L in wastewater and in surface water and 200 gc/g in sediment and in shellfish. Results below the LOD were considered 'negative' and samples with concentration below the LOQ were considered 'detected'.

Linear regression analysis was performed to estimate the relationship between the physico-chemical characteristics of water (pH, turbidity) and viral titres. These relationships were computed separately for the three source types: wastewater influents, effluents and surface waters. To assess the relationship between viral titres, a chi-square test was first carried out based on presence/absence, absence being identified with negative. Tests were carried out for source types both individually and combined. Correlations between viral titres were then computed, restricting the analysis to those samples with significant (i.e. non-negative/detected) counts, and using data from both individual and combined source types. We used a $\log_{10}$ transformation of all non-negative/detected virus counts in the statistical analysis. All statistical analyses were performed using the R software version 3.3.2 (R Core Team, 2013).

3. Results

3.1. Method validation and quality control

For method validation, treated and untreated wastewater samples were collected at the four WWTPs during a NoV outbreak that affected the area. As the viral concentration was expected to be high, these samples were not spiked with viruses but screened for human AdV, JCV, BKV, NoVGI/GII, SaVGI, HAV and HEV. High concentrations of AdV and JCV (10^4–6×10^5 gc/L) were noted in all samples (Fig. 2). High concentrations of BKV were found in samples derived from Ganol, Llanrwst and Tal-y-Bont, but BKV was not recovered from samples from Betws-y-Coed. The concentration of NoVGII was also extremely high in all samples and a low concentration of NoVGI was also noted. In the treated

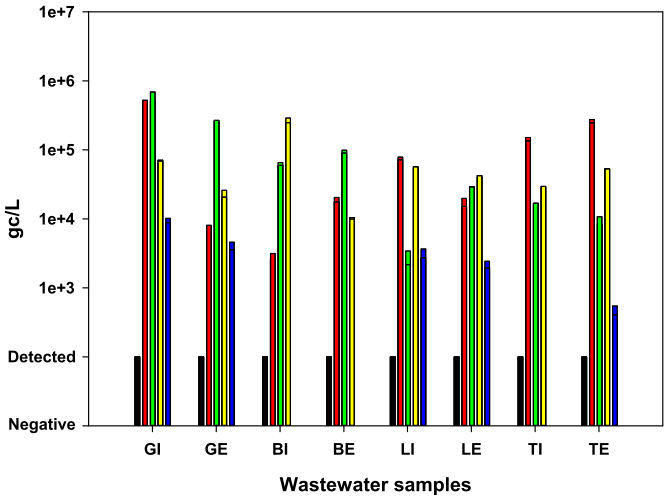
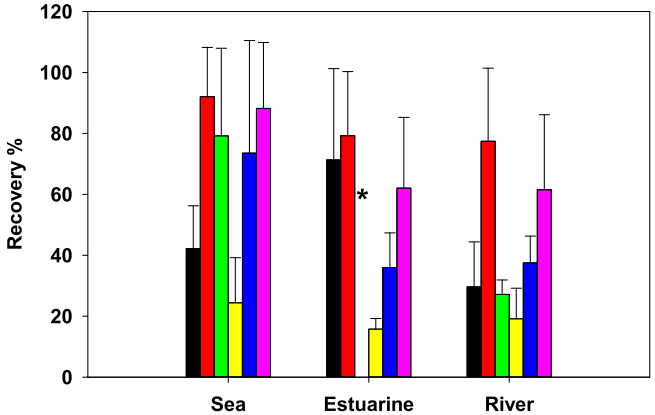


Fig. 2. Validation of the wastewater concentration method: Concentration of NoVGI (black), NoVGII (red), AdV (green), JCV (yellow) and BKV (blue) in influent and effluent samples derived from Ganol (GI and GE), Betws-y-Coed (BI and BE), Llanrwst (LI and LE) and Tal-y-Bont (TI and TE) WWTPs following the two-step water concentration method. Samples were collected during a local gastroenteritis outbreak. Bars represent the individual results of duplicate samples. Detected: indicates positive samples below the limit of quantification (LOQ) of 200 gc/L.

wastewater samples derived from the Ganol WWTP, a 0.3–1.7 $\log_{10}$ reduction in viral concentrations was observed. Little or no reduction was found in wastewater effluent from the other sites, relative to the influent. Indeed, in some instances the viral titre in the effluent was greater than observed in the influent.

Seawater, estuarine water and river water samples, 10 L each in triplicate, were spiked with approx. 10^6 gc NoVGII, AdV type 40, SaVGI and MgV. The seawater and river water samples were also spiked with approx. 10^6 gc HAV. Overall, the viral recovery ranged from 12% to 115% with the lowest recoveries observed for SaVGI (12.0–38.5%) and NoV (12.5–57.1%) followed by HAV (22.4–110.7%), MgV (25.3–114.9%) and AdV (33.4–105.4%; Fig. 3). The concentrated water samples were assayed with PGM-MBs to enumerate intact viral particles. The recoveries for different water types varied between 50% and 105%, which were



	Sea	Estuarine	River
pH	8.05	6.91	7.5
T (NTU)	6.37	11.74	3.2
K (mS/m)	40.7	18.88	0.06

Fig. 3. Water quality and the recoveries of NoVGII (black), NoVGII after PGM assay (red), HAV (green), SaVGI (yellow), MgV (blue) and AdV (pink) in three surface water sample types spiked with known concentration of the enteric viruses following the two-step water concentration method. *HAV was not used for spiking. Error bars represent standard deviation (n = 3).

higher than the recoveries observed using direct nucleic acid extraction (Fig. 3).

As a whole process control, MgV was added in known concentration to the samples prior to water concentration or the elution of viruses from sediment and shellfish, and recovery percentiles were determined (Table 1). Results showed that MgV could be efficiently recovered from the spiked samples. The wash samples collected between TFUF runs were all negative for the viruses tested suggesting no cross-contamination. To determine the efficiency of the PGM assay, the surface water samples spiked with enteric viruses were tested. Using the PGM assay, 62–100% recoveries were achieved (Fig. 3). To assess the rate of false positive results, concentrates considered negative were spiked with a known concentration of NoVGII RNA and the RNA was quantified after PGM treatment. The NoVGII RNA recovery was <0.01% suggesting very little or no RNA binding to the PGM-MBs.

3.2. Surveillance of enteric viruses in wastewater

High titres of AdV and JCV were noted in all wastewater influent and effluent samples (Fig. 4; Table 2). In total, 90% of the wastewater influent samples contained AdV and the same number of positives was observed for JCV. Slightly higher concentrations were observed during spring (from March to June 2017) compared to the concentrations in the rest of the samples (Fig. S1A). For wastewater effluent, 87% and 90% of the samples were positive for AdV and JCV, respectively. No seasonal patterns were evident, however a few peaks were observed in the samples taken at Betws-y-Coed (Fig. S1C).

Lower abundance was observed for the RNA viruses studied. Overall, 35%, 62% and 27% of the influent samples and 38%, 49% and 10% of the effluent samples were positive for NoVGI, NoVGII and SaVGI, respectively. NoVGI peaked during the spring months (from March to May 2017), whereas two peaks in NoVGII concentrates were noted; one during October–December 2016 and another during the spring months (Fig. S1B, D). SaVGI was only detected between March and August 2017. The abundance and concentration of RNA viruses was much higher at the largest WWTP (Ganol) compared to the smaller treatment works. HAV and HEV were not detected in any of the samples.

Overall, up to 2 $\log_{10}$ lower viral concentrations were noted in the effluent samples compared to the influent samples from all WWTPs. Of the 10 linear regressions between viral titres and pH or turbidity, (two source types; five viruses), a significant slope ($p < 0.05$) was only found for one turbidity relationship (NoVGII in wastewater influent) while no pH relationships were apparent. The chi-square tests based on presence and absence gave one significant ($p < 0.05$) result from 20 tests. There was some weak evidence of association between some viruses in WWTP effluent when both members of a pair were present in appreciable quantities. The pairs showing significant ($p < 0.05$) correlations were (NoVGI, SaVGI), (NoVGI, HAdV) and (AdV, JCV). For these water sources, the data showed little evidence of a relationship between viral titres and pH or turbidity, or between viral titres, once source type and location had been accounted for.

Table 1
Mengovirus recoveries observed in different sample types derived from the one-year surveillance of enteric viruses.

	Recovery %	SD
Wastewater influent (n = 52)	38.23	12.75
Wastewater effluent (n = 39)	47.33	34.12
Surface water (n = 52)	59.46	40.60
Sediment (n = 39)	61.31	37.06
Shellfish (n = 26)	68.85	34.83

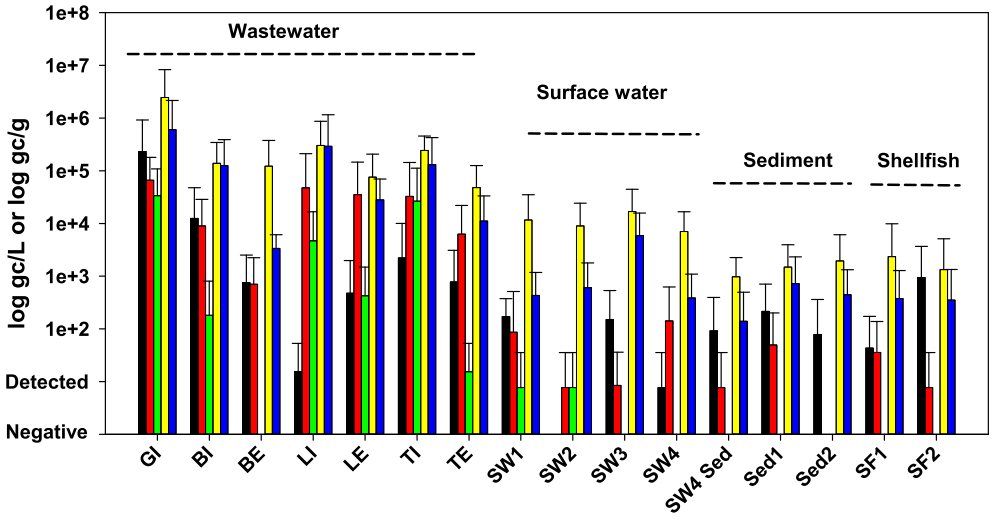


Fig. 4. Arithmetic mean concentration of NoVGI (black), NoVGII (red), SaVGI (green), AdV (yellow) and JCV (blue) observed in wastewater, surface water (SW), sediment (Sed) and shellfish (SF) samples over 12 months. Negative samples were estimated as 0 gc/L or gc/g and positive samples with concentration below LOQ were estimated as 100 gc/L or gc/g for calculations. Error bars represent standard deviation (n = 13).

3.3. Surveillance of enteric viruses in surface water

Compared to the wastewater samples lower abundance and viral concentrations were observed in the surface water samples (Fig. 4 and Fig. S2). The viral concentrations observed in the surface water samples were 1–4 $\log_{10}$ lower than those observed for the wastewater samples. Most surface water samples contained AdV, however, fewer samples were positive for JCV (88% vs 65%). Interestingly, almost all SW3 samples were positive for JCV, whereas the JCV was less abundant in the other surface water samples (Fig. S2). The ratio of the samples positive for the RNA viruses was also low; 10%, 13% and 4% of the samples were positive for NoVGI, NoVGII and SaVGI, respectively. Noroviruses were present in the samples taken in September and November 2016 at SW1 and SW4, in January 2017 at SW2 and in February and May 2017 at SW1 and SW3. In general NoVGI concentrations exceeded the NoVGII concentration in the surface water samples. SaVGI was only present in samples taken at SW1 and SW2 in September 2016. HAV and HEV were not detected in any of the samples. No significant regression relationship was found between viral titres and either pH or turbidity. The chi-square test gave no significant ($p < 0.05$) test result, nor were there any significant correlations ($p < 0.05$) between viral titres.

3.4. Surveillance of enteric viruses in sediment and shellfish

In the sediment and shellfish samples, the proportion of samples positive for AdV was similar to the amount observed in the water samples (82% and 85% in sediment and shellfish samples, respectively), however, the abundances of the other target viruses were considerably lower (Fig. S3; Table 3). The abundance of JCV was lower than in the water samples, 38% of the sediment and 35% of the shellfish samples were positive. Interestingly, in sediment and shellfish samples, the NoVGI was more abundant than NoVGII (18% vs 8% in sediment and 19% vs 12% in shellfish samples). NoV concentrations peaked during September–November 2016 and January–March 2017 in the sediment and shellfish samples. All sediment and shellfish samples were negative for HAV, HEV and SaVGI. There were no significant chi-square test results or correlations between viral titres for these source materials.

3.5. Norovirus capsid integrity

A PGM assay was used to assess the viral capsid integrity in 43 concentrates positive for NoVGI and 54 concentrates positive for NoVGII. Results showed that the 83% of the wastewater influent samples were positive for NoVGI using the PGM assay and all influents were positive

Table 2
The measured pH, turbidity (T), conductivity (K) values with standard deviations (SD) and the number of samples positive for norovirus GI and GII (NoVGI, NoVGII), sapovirus GI (SaVGI), adenovirus (AdV) and JC polyomavirus (JCV) in each sample type. Blank cells: parameter not measured.

	pH n = 13	T (NTU) n = 13	K (mS/m) n = 12	NoVGI n = 13	NoVGII n = 13	SaVGI n = 13	AdV n = 13	JCV n = 13
GI	7.6 ± 0.2	189 ± 96		8	9	5	12	13
BI	7.3 ± 0.3	157 ± 86		4	8	2	11	11
BE	7.1 ± 0.4	35 ± 114		4	5	0	10	11
LI	7.5 ± 0.6	209 ± 158		2	7	2	11	11
LE	6.8 ± 0.3	23 ± 17		4	8	2	12	13
TI	7.1 ± 0.4	164 ± 226		4	8	5	13	12
TE	7.0 ± 0.3	9 ± 11		7	6	2	12	11
SW1	7.4 ± 0.5	7 ± 17	0.05 ± 0.03	2	3	1	11	7
SW2	7.3 ± 0.4	4 ± 3	0.08 ± 0.02	0	1	1	11	7
SW3	7.2 ± 0.4	6 ± 4	0.63 ± 1.60	2	1	0	12	12
SW4	7.1 ± 0.4	17 ± 19	96.76 ± 188.47	1	2	0	12	8
Sed1				4	2	0	10	6
Sed2				1	0	0	11	6
Sed4				2	1	0	11	3
SF1				2	2	0	11	4
SF2				3	1	0	11	5

Table 3
Number of positive samples assessed using direct extraction and PGM assay.

Virus type	Sample type	Direct extraction	PGM assay
NoVGI	Influent (n = 52)	18	15
	Effluent (n = 39)	14	8
	Surface water (n = 52)	4	1
	Sediment (n = 39)	7	1
NoVGII	Influent (n = 52)	29	29
	Effluent (n = 39)	16	12
	Surface water (n = 52)	5	2
	Sediment (n = 39)	4	0

for NoVGII (Table 3). Fewer effluent samples were positive for NoVGI (57%), whereas 75% were positive for NoVGII. Few surface water and sediment samples were positive for NoV (0–25%) following the PGM assay. In general, the viral concentrations observed after PGM assay were lower than those observed following direct extraction (Table 3, Fig. S4). The only exception was the batch of wastewater influent samples tested for NoVGII; 19 samples of the 29 tested showed higher concentration of NoVGII by PGM assay than by direct extraction.

4. Discussion

Considering the large number of food- and waterborne disease outbreaks associated with enteric viruses, a greater understanding of the fate and transport of these viruses in the environment from source to point of use is needed. The aim of this study was to investigate viral contamination at source (WWTPs) and in the receiving river and estuary, including treated and untreated wastewater, surface water, sediment and shellfish samples, over an annual cycle. The methods used for sample concentration and quantification were carefully validated, thus enabling the accurate estimation of viral titres. For the quantification of the viruses q(RT-)PCR was used, however, this approach does not address viral integrity and infectivity. Therefore, to evaluate viral degradation, a capsid integrity assay using PGM-MGs was also used. We targeted viruses that are commonly associated with waterborne and shellfish-borne outbreaks (NoVGI, NoVGII and HAV), emerging viruses that have been shown to be associated with outbreaks but rarely or not found in UK waters (SaV and HEV) and potential indicator viruses (AdV and PyV). Viral concentrations have been compared with the pH, turbidity and conductivity of the water samples, however, little or no correlation was found, most likely due to a small sample size. A more focused monitoring campaign would be necessary to accurately describe the potential correlations between physico-chemical properties and microbial quality.

4.1. Evaluation of the methods used for the detection of viruses

In this study we validated a two-step concentration method for the detection and quantification of viruses in surface water and wastewater. The method includes a TFUF step using a 100 kDa membrane to reduce sample volume of between 1 and 10 L to approx. 50 mL. TFUF has been widely used to concentrate a variety of microbial pathogens in water, e.g. *Cryptosporidium*, *Giardia*, *Salmonella*, *Escherichia*, *Enterococci*, *Clostridium* spp. together with viruses (Francy et al., 2013; Kahler et al., 2015; Liu et al., 2012). To assess cross-contamination between samples, the solutions used for the final wash step between experiments were also checked for enteric viruses. No viruses were detected in those solutions suggesting that the membranes can be reused. The second step of our concentration method involves a beef extract elution and a PEG precipitation. PEG precipitation has been used as a secondary concentration step following TFUF (Kahler et al., 2015). The elution step allowed the viral particles to detach from solid matter in the primary concentrate and hence improve the efficiency of the PEG precipitation. For viral detection and quantification, nucleic acids were extracted and quantified using q(RT-)PCR.

For method validation, river, estuarine and seawater samples (10 L each) were spiked with a mix of viruses including NoVGII, SaVGI, HAV, AdV and MgV in triplicates. The recovery rates varied between 12 and 100% (Fig. 3) suggesting that the method is suitable for the recovery of viruses. Wastewater samples were not spiked with enteric viruses for method validation due to the high abundance of target viruses in wastewater. Instead, wastewater influent and effluent samples were collected at four locations during a NoV outbreak in the area. The high concentration of AdV, JCV, BKV and NoVGII agree with viral concentrations usually found in wastewater (Kitajima et al., 2014; Tran et al., 2015). Interestingly, BKV was only found in high concentrations in three out of four sites, suggesting it is less abundant than JCV or the other target enteric viruses. As the aim of the inclusion of polyomaviruses in this study was to find potential indicators for viral wastewater-derived contamination in the environment, BKV was excluded and only JCV was targeted during the surveillance experiment. Interestingly, in some cases, higher viral concentrations were observed in the treated effluent wastewater samples than in the corresponding untreated influent samples. The results were consistent in diluted nucleic acid extracts, hence inhibition during detection was unlikely. As the influent and effluent samples were taken at the same time, the variations in viral titres in effluent were probably due to the diurnal changes in wastewater quality.

For the elution of viral particles from sediment and shellfish, established methods were used. The elution-concentration method used for sediment samples has been shown to sufficiently recover NoVGII, HAV, rotavirus and poliovirus from estuarine sediment (Farkas et al., 2017a; Lewis and Metcalf, 1988). For shellfish samples the ISO/TS15216-1 standard was used. This assay was developed for the quantification of RNA of NoVGI, NoVGII and HAV in shellfish digestive tissue and has been widely used by researchers and reference laboratories worldwide.

For further validation of the methods and for quality control, all samples collected were spiked with a known concentration of MgV. Mengovirus was recovered in all samples with rates exceeding 10% (Table 1), which meets the quality control requirements of standardised methods. For instance, the ISO/TS15216-1 method used for the detection of enteric viruses in shellfish requires 1% recovery of the process control. The Method 1615 provided by the US Environmental Protection Agency (EPA) for the concentration of environmental and drinking water samples for the quantification of enteric viruses allows 5–200% recoveries for the process control (Fout et al., 2010). Hence, the recovery achieved in this study and the usefulness of our method for difficult water samples suggests that the method can be standardised and used for routine monitoring.

In this study, qPCR and qRT-PCR were used for the primary detection and quantification of the target viruses. As there is no culture-based assay that can be used for the monitoring of many enteric viruses, e.g. human NoV and SaV strains, q(RT-)PCR assays are the most frequently used techniques for the enumeration of pathogenic viruses in the environment. In order to assess viral infectivity, genome and capsid integrity assays have been established and in this study we evaluated the usefulness of a histo-blood group antigen affinity assay using PGM-MBs that have the ability to bind to NoV surface antigens (Dancho et al., 2012; Tian et al., 2008). The subsequent qRT-PCR therefore only detects RNA derived from intact viral particles. In order to validate the assay, the surface water concentrates spiked with NoVGII were tested using PGM-MBs. In all instances, higher recoveries (60–100%) were achieved using this assay than when direct extraction was used (Fig. 3) suggesting that the use of PGM eliminated some proportion of organic matter that inhibits nucleic acid extraction and qRT-PCR. This correlates with previous findings where a 1–2 $\log_{10}$ increased sensitivity was observed when using the PGM assay on food samples (Tian et al., 2008). Further validation using NoV RNA shows that the assay excludes free RNA. Hence, the PGM assay is suitable for the recovery of intact viruses and may be used as an additional purification step prior to RNA extraction when NoV is being monitored.

4.2. Surveillance of viruses

During our surveillance of enteric viruses in wastewater and in the environment, no HAV or HEV were found. These strains are not abundant in the UK except for a few sporadic cases, and during this study no HAV- or HEV-illness was reported to the national public health agencies. High titres of NoVGI, NoVGII, SaVGI, AdV and JCV were found. The highest concentrations were found in wastewater influent and effluent samples. Viral titres, and the number of positive samples, significantly reduced in surface water, sediment and shellfish samples (Table 2). These viruses are frequently found in wastewater and surface water worldwide (Tran et al., 2015), however, to our knowledge, this is the first study detecting SaV in wastewater and river water in the UK. SaVGI was present in multiple wastewater influent and effluent samples over March–June 2017 at high concentrations and was detected at low concentrations in two river water samples in September 2016. No SaV-related outbreaks were reported during the period of this study. However, SaV-related symptoms are very similar to NoV symptoms and hence SaV-related illnesses may not be reported. SaVGI was not found in any of the sediment or shellfish samples that may suggest relatively rapid die-off in the environment.

NoVGI and NoVGII were frequently found in all sample types throughout the study. In general, the highest NoVGII concentration was noted in November 2016 and peaks of NoVGI titres were observed during the spring months of 2017 in all sample types. These findings correlate well with the sporadic outbreaks noted in the monitoring area during October–December 2016 and February–May 2017 (Public Health Wales, personal communication). Similar correlations were found in a previous study on NoV titres in river water vs. gastroenteritis outbreaks in France (Prevost et al., 2015). The peak NoV concentrations varied between 10^5 and 10^7 gc/L in influent samples and the highest concentrations were noted in samples derived from the largest WWTP (Ganol). These findings agree with previously reported NoV concentrations (10^2–10^7 gc/L) in untreated wastewater (Grøndahl-Rosado et al., 2014; Kitajima et al., 2014; Rusiñol et al., 2015), however, higher NoV concentrations have also been noted (Gerba et al., 2017). The differences in viral titres at different sites may be due to the differences in population served, site size and weather conditions. Only a slight reduction was observed in the effluent samples. These results correlate with previous findings that suggested a 1–3 $\log_{10}$ reduction in enteric virus concentrations during wastewater treatment (Kitajima et al., 2014; Qiu et al., 2015). Considerably lower NoV abundance was observed in surface water samples with peaks of 10^3 gc/L and in most cases NoVGI was more abundant than NoVGII. Only a few sediment and shellfish samples were positive for NoV with concentrations up to 10^3 gc/g. Interestingly, more sediment and shellfish samples were positive for NoVGI than for NoVGII (Table 2). The high NoVGI concentrations and abundance suggest that NoVGI is more persistent in the environment than NoVGII. Previous studies show that even though NoVGII is the most prevalent genotype globally, NoVGI is frequently found in environmental water and in shellfish and associated with illnesses (Kageyama et al., 2004; Le Guyader et al., 2006; Maalouf et al., 2010; Pérez-Sautu et al., 2012). Furthermore, NoVGI has been found to be more persistent to wastewater treatment procedures, UV and chemical disinfection treatments than NoVGII (Duizer et al., 2004). These findings suggest NoVGI is more persistent in the environment than NoVGII, however comparative studies are needed to fully understand the differences in the behaviour of these genotypes in the environment. The NoV found in shellfish samples may pose a potential public health hazard. According to the microbiological classification of shellfish harvesting waters and marketing requirements for human consumption (EC directive 91/492) the two monitored shellfish harvesting areas are Class B sites and shellfish from these areas should be depurated before commercial sale. Nonetheless, the contaminated shellfish may cause illness in people consuming shellfish as viral titres take longer than those of bacteria to be reduced in shellfish (Dore and Lees, 1995).

AdVs and PyVs have been proposed as a potential indicator for wastewater contamination (Bofill-Mas et al., 2006; Hewitt et al., 2013; Pina et al., 1998; Rachmadi et al., 2016). These viruses are usually carried asymptomatically within the human population and are frequently found in wastewater in high concentrations, and appear to be resistant to wastewater treatment. Their persistency and behaviour in the environment are assumed to be similar to those of pathogenic enteric viruses and hence reflects faecal contamination more accurately than traditional indicators, e.g. bacteriophages (Lin and Ganesh, 2013). Supporting previous findings mentioned above, our study also showed high abundance of these viruses in wastewater and in the environment. Overall, 87% of the samples were positive for AdV and 67% of the samples were positive for JCV (Table 1). Interestingly, while AdV was frequently found in all sample types, JCV was more prevalent in wastewater and less frequently found in surface water, sediment and shellfish. The only exception was SW3 (sampling point close to the discharge point of Llanrwst WWTP), where JCV was found in 12 of the 13 samples taken. These findings suggest that AdV may be more persistent in the environment and reflect the behaviour of persistent enteric viruses, whereas JCV may degrade more rapidly and its survival is more consistent with less stable pathogens. These findings correlate with previous results showing high AdV concentrations over a year in wastewater and surface water in Norway (Grøndahl-Rosado et al., 2014) and in Sweden (Rusiñol et al., 2014) and in a Mediterranean catchment (Rusiñol et al., 2015). No seasonal changes were observed in the concentration of AdV and JCV in any sample type except wastewater influent, where slightly higher concentrations were observed during spring-summer than during autumn-winter. The high number of viruses may be due to the greater number of tourist visitors to the area during the warmer months of the year, in agreement with previous research showing a positive correlation between the level of urbanisation and environmental AdV and JCV concentrations (Rusiñol et al., 2014).

In general, we observed that high NoV and SaV concentrations tended to be associated with high AdV and JCV concentrations. However, it was very frequently the case that high AdV and JCV concentrations were associated with negative NoV and SAV results. Due to the high AdV and JCV titres in all sample types and the lack of seasonality, AdV and JCV may be a suitable microbial source tracking tool for assessing human wastewater contamination in the environment (Rusiñol et al., 2014) without representing specific pathogenic viruses.

4.3. Evaluation of viral degradation in the environment

Due to the lack of reliable in vitro culturing method for NoV that can be routinely used, viral survival was assessed using a PGM-based capsid integrity assay. The assay has been shown to sufficiently distinguish NoVs with intact capsids in a range of matrices including water (Cannon and Vinjé, 2008) and fresh food products (Tian et al., 2008) and have been used to model viral inactivation (Kingsley et al., 2014; Li and Chen, 2015; Dancho et al., 2012). In our study, the wastewater, surface water and sediment samples positive for NoV when direct extraction was used were assayed using PGM-MBs. The shellfish samples were not suitable for the assay as they were pre-treated with proteinase K for virus elution and the enzyme will degrade viral proteins in the samples. The results of the PGM assay suggested little viral degradation in the wastewater influent samples. In the majority of the samples, higher NoVGII concentrations were observed with the PGM assay than without it (Fig. S4), suggesting that the assay is suitable for removing inhibitors as shown elsewhere (Tian et al., 2008). In contrast, lower concentrations of NoVGI were detected when the PGM assay was used compared to direct extraction in all sample types including wastewater influents. This may indicate that the NoVGI strains found in the environment may have a different binding affinity to PGM. This theory is supported by the study of Hutson et al. (2004) that revealed strain-dependent differences in the binding pattern of NoV to antigens present

in PGM. Furthermore, previous studies also observed that NoVGI have lower binding affinity to PGM than NoVGII (Tian et al., 2010).

The number of positive samples, as well as the viral concentrations, was slightly lower in wastewater effluent samples when the PGM assay was used suggesting limited viral degradation in the samples. A few surface water and sediment samples were also positive with the PGM assay for both NoVGI and NoVGII confirming viral degradation in the environment. Nonetheless, the positive samples indicate a potential public health threat and the presence and concentration of intact pathogenic viruses needs to be further investigated.

The PGM assay is a rapid and simple method for the extraction of NoV particles from environmental samples. However, it may also detect semi-degraded viral particles (i.e. RNA attached to decayed capsid) and hence further validation is needed. Recently an in vitro human NoV culture method has been established using stem cell-derived human enteroids (Ettayebi et al., 2016) and that assay may be suitable for the validation of capsid integrity assays. Nonetheless, our results show that the PGM assay provides a more accurate estimation on viral titres than direct extraction and hence public health risks associated with waterborne and foodborne NoV can be better estimated.

The methods validated in this study are suitable for the identification, quantification and surveillance of viruses in the aquatic environment. The surveillance revealed that high titres of intact and potentially infectious enteric viruses enter the environment via wastewater discharge. Viral titres correlate with outbreaks affecting the population of the study area. An emerging pathogen (SaV) was detected and quantified for the first time in UK waters along with indicator viruses (AdV and JCV) that inform on the spread of wastewater-associated pathogens in the environment. Furthermore, the survival of NoVs has also been assessed. However, further research is needed to discover the strain-related differences in viral persistence. The data can be applied to inform predictive models for the transport of enteric viruses in water and to improve current viral risk assessment.

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Research data

Metadata is available at the Environmental Information Data Centre (EIDC, www.eidc.ceh.uk). Doi:https://doi.org/10.5285/5d19f6e2-1383-41ed-92d2-138d95bf4c72.

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